

# Structural Characterization of Self-Assembled Polypeptide Films on Titanium and Glass Surfaces by Atomic Force Microscopy

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Chemically modified biomaterial surfaces (titanium and glass) covered with polyelectrolyte self-assembled films formed by the alternating adsorption of cationic poly-L-lysine (PLL) and anionic poly-L-glutamic acid (PGA) were structurally characterized by atomic force microscopy. Complementary information concerning the thickness and layer-by-layer growth of the films was provided by optical waveguide light-mode spectroscopy. The frequently used *ex situ* and the rarely used *in situ* build-up methods were compared. Important aspects of the industrial applicability of these films, their stability in time, and possible differences in their morphology were investigated. The films revealed a granular pattern, with grain diameters of  $270 \pm 87$  nm for glass (up to 8 bilayers) and  $303 \pm 89$  nm for titanium (up to 10 bilayers), independently of the build-up procedure. Both surfaces displayed a rehydration capability, the titanium surface exhibiting a better stability in time. The high roughness values observed at acidic or basic pH are related to the degree of ionization of PGA and PLL.

## 1. Introduction

This work addresses the implantological problem of how to make the surface of a biomaterial biocompatible. The success and long-term prognosis of endosseous implants depend primarily on the anchorage of the implant in the host bone,<sup>1</sup> i.e., the osseointegration.<sup>2</sup> The good osseointegration of alloplastic materials in contact with bone tissue requires the formation of strong links between the biomaterial and the surrounding bone tissue.<sup>3</sup>

Thanks to their good integration, physical properties and predictable, long lifetime, titanium (Ti) and its alloys are nowadays widely used in implantology.<sup>4,5</sup> The surface of an implant is in immediate contact with the biological medium; its interfacial characteristics (topography, chemistry or surface energy) and surface improvement therefore play essential roles in biointegration.<sup>3,6</sup> One solution with which to improve the surface of an implant has proved to be modification of the surface chemistry by the formation of polyelectrolyte (PE) multilayer films that are nanometer-sized and can be biofunctionalized toward targeted properties. This is certainly one of the driving forces for the increasing interest in this field. PE film coatings modify the solid/liquid

interface in such a way as to ensure a proper environment for the adsorption of proteins, which is a key factor determining the interactions between the implant and the surrounding medium. Thus, the distribution of functional groups on the surface of the biomaterial and the macromolecular structure are properties that it is essential to study,<sup>7–9</sup> and this was our main aim.

PE multilayers are formed by the alternating adsorption of polycations and polyanions from aqueous solution onto a charged, solid surface.<sup>10</sup> The alternating adsorption technique has been successfully applied in different fields of science, as a consequence of its numerous practical applications. It can be automated, it involves the use of aqueous solutions, it is environment-friendly, and various substrates can be covered with films of readily variable thickness.<sup>11,12</sup>

Within the large number of candidate PEs, we were interested in biodegradable ones; accordingly, we studied multilayers formed by the alternating adsorption of polycationic poly-L-lysine (PLL) and polyanionic poly-L-glutamic acid (PGA). Previously published results<sup>13–15</sup> allow the expectation that PE multilayers should multiply the possibilities for the induction of cellular reactions if the cells are able to respond to bioactive molecules, e.g., signal transduction molecules embedded in the film.

It has been suggested that the self-assembling approach offers many advantages in the construction of PE coatings. We were interested in the validity of this for the chosen (PLL/PGA) system, i.e., in the effects of the applied procedure,

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the substrate type, and the topology on the film stability. Thus, we investigated the microstructure of self-assembled films deposited on solid surfaces that might serve as future biomaterials. The build-up of PE multilayers was followed in situ by optical waveguide light-mode spectroscopy (OWLS), and atomic force microscopy (AFM) was employed to visualize the PLL/PGA coatings after each new deposition and to characterize them structurally. To date, only a few studies have been carried out with this method, mainly by means of an ex situ build-up of films<sup>15–17</sup> or partial in situ layering.<sup>8</sup>

We first compared the effects of the two different sample preparation methods on the morphology of the coatings obtained. Besides the glass substrate used in the cited works, our study also included PLL/PGA build-up on a Ti substrate, which is often used as an implant in medical interventions. Thus, PE films were deposited onto Ti and glass substrates by sequential adsorption methods in two ways: the build-up was performed either in situ, in the liquid cell of the atomic force microscope (in situ samples), or ex situ, outside the microscope, by an automatic immersion technique (dry samples). Possible differences in film surface characteristics between the two build-up procedures were investigated and the stability in time of these films was also examined, this being an important aspect as concerns industrial applicability. Further, the effects of pH and rehydration were evaluated: for such films to be applicable in practice, hydration should not modify or destroy the applied coating. A controlled rehydration process allows delivery of the dry implant, which can be hydrated directly during the surgical process.

## 2. Materials and Methods

**2.1. Materials.** Microscope cover glass disks (Marienfeld, Germany) 14 mm in diameter were cleaned in a 3% solution of Hellmanex (Hellma GmbH) and then in a 0.1 M HCl solution for 15 min at boiling temperature, and finally rinsed in pure distilled water. Ti sample disks 1.5 mm thick and 15 mm in diameter were cut from commercially pure (grade IV) Ti rods (Dentaurum, Germany) used for the fabrication of dental implants. To clean the surface, the samples were exposed to treatment in acetone or ethanol for 15 min in an ultrasonic bath and then sonicated in water three times for 10 min. PE solutions were prepared in an aqueous buffer solution of 25 mM TRIS (tris(hydroxymethyl)aminomethane, Sigma), 25 mM MES (2-(*N*-morpholino)ethanesulfonic acid, Sigma), and 100 mM NaCl (Fluka), pH 7.4. When the pH dependence was investigated, the buffer was adjusted to pH 5.0 or 9.0, with HCl or NaOH solution, respectively. The PE films were formed by the alternate adsorption of cationic PLL ( $M_w = 30\,000$ – $70\,000$ , Sigma Aldrich, P-2636) and anionic PGA ( $M_w = 50\,000$ – $100\,000$ , Sigma Aldrich, P-4886). The PE concentration was in all cases 1 mg/mL. Solutions were prepared with ultrapure water (Milli-Q-plus system, Millipore), and all buffer solutions were filtered before use.

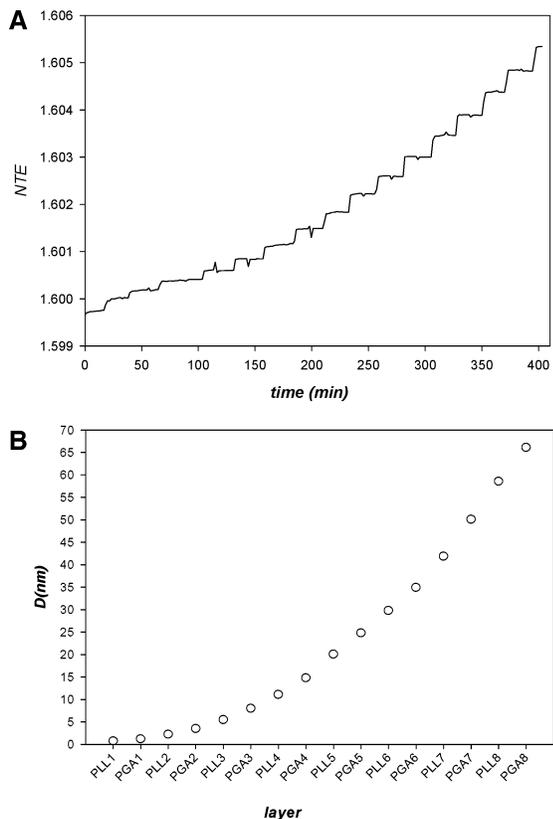
**2.2. Optical Waveguide Light-Mode Spectroscopy.** OWLS is an optical technique, based on the confinement of a laser beam in a high refractive index layer of  $\text{Si}_{0.8}\text{Ti}_{0.2}\text{O}_2$

(waveguide) by means of a grating coupler. The experimental details of the method are given elsewhere.<sup>18,19</sup> The adsorption of a film onto such a waveguide perturbs the evanescent field and leads to changes in the effective refractive indices of the transverse electric ( $N_{\text{TE}}$ ) and transverse magnetic ( $N_{\text{TM}}$ ) modes. OWLS records with high precision ( $\Delta N \sim 10^{-5}$ ) the changes in  $N_{\text{TE}}$  and  $N_{\text{TM}}$  up to a film thickness of roughly 350 nm. The measured  $N_{\text{TE}}$  and  $N_{\text{TM}}$  values depend on the refractive index profile of the film deposited on the waveguiding layer. By means of the simultaneous measurement of two modes (p-polarized and s-polarized), the thickness and the refractive index of the adsorbed layer can be calculated. The mode equations were resolved without a thin layer approximation, and the structural parameters, i.e., the refractive index and thickness ( $n_A$  and  $d_A$ ) of each deposited layer, were obtained.<sup>17</sup> The PE multilayer film was then built up as follows: first, a 1 mg/mL PLL solution in 25 mM MES, 25 mM TRIS, and 100 mM NaCl, pH 7.4 was injected for 15 min. Then, PLL/PGA, PLL/PGA/PLL, ... architectures were built up progressively up to (PLL/PGA)<sub>8</sub>. PE depositions were always separated by a 15-min long rinsing step.

**2.3. In Situ Build-Up of the PE Film in the Fluid Cell of the Atomic Force Microscope.** The glass or Ti substrate was placed into the Nanoscope (Digital Instruments, Santa Barbara, USA) liquid cell closed by a silicone O-ring. The closed system joint for the liquid cell was described previously.<sup>20</sup> Buffer solution was then flowed through this system. PLL was injected first and left to adsorb for 15 min. After three rinses with buffer solution, PGA was injected in the same way. Images were taken 15 min after the last rinsing step. In situ imaging was performed following each deposition from the 4th layer of PLL to the 8th layer of PGA.

**2.4. Ex Situ Build-Up of the PE Film outside the Atomic Force Microscope.** Both Ti and glass samples were placed in a special holder and were immersed alternately in PLL and PGA solutions by an automatic immersion device (Department of Colloid Chemistry, University of Szeged) at a constant speed. During each immersion period, the samples were kept in the solution for 15 min. Between two dips, the samples were rinsed three times with buffer solution. After formation of the last layer, the films (6, 8, or 10 bilayers) were rinsed in buffer and dried in a mild nitrogen-flow (high purity). Film-coated samples that had been aged for up to 1 month on the glass and for up to 2 months on the Ti substrate were studied by AFM. Rehydration was performed by wetting 6 week old samples with buffer solution for at least 1 h.

**2.5. AFM Measurements.** The different surfaces were subjected to structural characterization with a Nanoscope III atomic force microscope (Digital Instruments, Santa Barbara, CA). Silicon nitride tips (type MSCT-AUHW) were purchased from Veeco Metrology Group (Santa Barbara, CA). Cantilevers with spring constants of 0.01 and 0.03 N/m were used. Measurements were performed both in air and in a liquid cell. Height, deflection, and friction images with areas of  $10 \times 10 \mu\text{m}$  and  $5 \times 5 \mu\text{m}$  were captured in contact mode, similarly as in other publications.<sup>15,16,20</sup> Lobo et al.<sup>21</sup> have pointed out that, for the granulated structures of certain PE films, the tapping mode did not yield a good image contrast



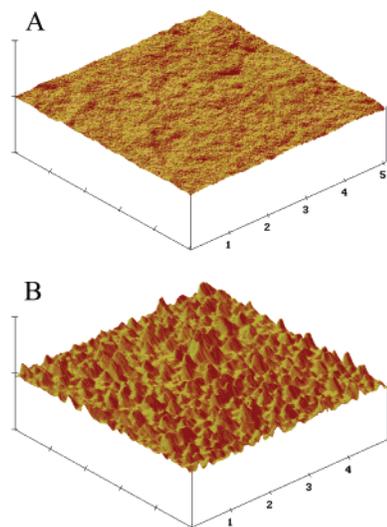
**Figure 1.** OWLS measurements. (A)  $N_{TE}$  values as a function of time and (B) film thickness ( $D$ ) values as a function of layer number.

for the grains. In these situations, the contact and friction force modes must be used. As we expected such granulated structures and possible clustering, especially at high pH, we employed contact mode AFM, furnishing the best image contrast with which to study the surface structure and morphology. However, to diminish the tip-sample interactions for in situ measurements, the AFM tips were silanized so as to transform the hydrophilic tip into a hydrophobic one, using a mixture of 10 mL of *n*-hexadecane, 5 drops of carbon tetrachloride, and 5 drops of *n*-octadecyltrichlorosilane (Sigma Aldrich). Silanization allowed the imaging of both negative and positive surfaces. Several scans of each surface were performed in order to check the reproducibility of the images and to reveal possible tip damage. All images were taken at a scan rate of 1.97 Hz, with a resolution of  $512 \times 512$  pixels, and 3D analysis was also performed.

The mean surface roughness ( $R_a$ ) of a film was obtained by averaging the surface roughness (at least 5 independent measurements), defined as  $R_a = 1/n \sum_{j=1}^n |Z_j|$ , the arithmetic average of the absolute values of the surface height deviations measured from the mean plane within the box cursor. The diameter of the grains was determined by performing section analysis in the Nanoscope software (Version 4.42, 1999).

### 3. Results and Discussion

**3.1. In Situ PE Deposition Measured by OWLS.** The step-by-step build-up of a (PLL/PGA)<sub>8</sub> film was recorded in situ by OWLS (Figure 1A). This experiment served as a first check on the chosen experimental conditions, and the time domains necessary for the reproducible alternating



**Figure 2.** 3D images of glass substrate and PE layers on it (in situ measurements). (A) Bare glass, data scale:  $x = 1 \mu\text{m}/\text{div}$  and  $z = 20 \text{ nm}/\text{div}$  and (B) (PLL/PGA)<sub>6</sub> PE film, data scale:  $x = 1 \mu\text{m}/\text{div}$  and  $z = 500 \text{ nm}/\text{div}$ .

adsorption of polypeptides forming the films to be further visualized by AFM. The thickness of the layers formed was calculated from the measured  $N_{TE}$  and  $N_{TM}$  values, as described in the Experimental Section. The final thickness obtained was about 70 nm. A plot of the layer-by-layer growth of the film thickness (Figure 1B) displays the well-known exponential growth of the PLL/PGA film governed by polypeptide diffusion in and out, as explained previously.<sup>17</sup>

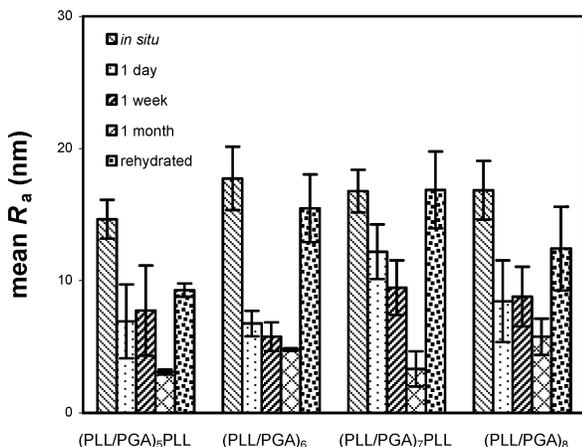
**3.2. PE Layers on a Glass Substrate.** PLL/PGA films comprising up to 8 bilayers (PLL/PGA)<sub>8</sub> were successfully layered during in situ build-up in the liquid cell of the atomic force microscope. The AFM images exhibited a granular pattern similar to those found in other systems.<sup>16</sup> The same pattern was observed for samples prepared ex situ. The grain diameter was  $270 \pm 87 \text{ nm}$ , in agreement with the values measured by Lavalle et al.<sup>17</sup> When not otherwise stated, the presented results will concern the film build-up under conditions close to physiological (150 mM ionic strength and pH 7.4).

The 3D images (Figure 2) demonstrated a significant morphological difference between the bare glass substrate and the (PLL/PGA)<sub>6</sub> film adsorbed on it.

For the bare glass, a mean roughness value of  $R_a = 0.5 \pm 0.2 \text{ nm}$  was obtained, whereas for (PLL/PGA)<sub>6</sub> the mean  $R_a$  was  $17.7 \pm 2.4 \text{ nm}$ , and for (PLL/PGA)<sub>8</sub>, it was  $16.9 \pm 2.2 \text{ nm}$ . For in situ deposition, the roughness was found to be independent of the number of layers (Figure 3).

PLL/PGA films were also built-up ex situ, by an automatic immersion method. Dry samples aged for 1 day, 1 week, or 1 month were produced in this way. AFM images were then recorded. The structural characterization of these samples and their comparison with the result of the in situ measurements provided valuable information. Figure 3 depicts the changes in mean roughness of (PLL/PGA)<sub>5</sub>PLL, (PLL/PGA)<sub>6</sub>, (PLL/PGA)<sub>7</sub>PLL, and (PLL/PGA)<sub>8</sub> films stored under dry conditions for up to 1 month.

In the in situ measurements, the roughness proved almost independent of the number of layers, whereas for the dry



**Figure 3.** Mean roughness values as a function of layer number and time for polypeptide films on a glass substrate. Rehydrated films were 6 weeks old.

samples, a significant variation was observed (see 1-day and 1-week old samples for the (PLL/PGA)<sub>6</sub> and (PLL/PGA)<sub>7</sub>PLL films). The dry samples with both 6 and 8 bilayers indicated a large decrease in roughness in time as compared with the in situ values, except for the (PLL/PGA)<sub>7</sub>PLL film, where a smaller, but still significant decrease was observed. The chart reveals noteworthy decreases in  $R_a$  in all cases for the 1-month-old samples.

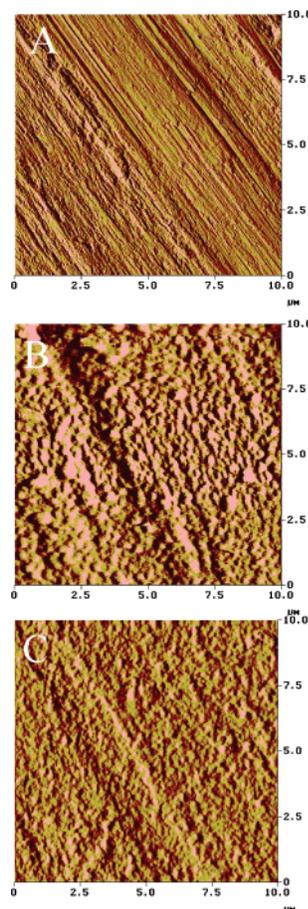
The PE films on a glass substrate also exhibited a significant time dependence, e.g., for the 1-day-old (PLL/PGA)<sub>7</sub>PLL film  $R_a$  was  $12.2 \pm 2.1$  nm, whereas after 1 month, it was  $3.3 \pm 1.3$  nm. Even after 1 day, a significant decrease in  $R_a$  was experienced, as observed for the (PLL/PGA)<sub>8</sub> films: the in situ value was  $16.9 \pm 2.2$  nm, whereas for the 1-day-old sample, it was  $8.4 \pm 3.1$  nm.

Upon rehydration of a 6-week-old dry sample, the roughness values almost reached those measured in situ, independently of the nature of the last layer. For the (PLL/PGA)<sub>6</sub> film, the mean  $R_a$  recovered to  $15.5 \pm 2.6$  nm, whereas that for the (PLL/PGA)<sub>7</sub>PLL film did so to  $16.9 \pm 2.9$  nm. The data prove that the PLL/PGA films possess a high capability to rehydrate and to recover their morphology when placed in a wet environment.

**3.3. PE Layers on a Ti Substrate.** PLL/PGA multilayers on Ti disks were also built up in situ in the liquid cell of the atomic force microscope. Typical AFM deflection images of the Ti substrate and (PLL/PGA)<sub>6</sub> and (PLL/PGA)<sub>8</sub> films may be seen in Figure 4. The original rough-machined surface of the Ti disk can still be discerned even when the surface is covered totally by a PE film.

PLL/PGA films were also built up on a Ti substrate by the automatic immersion method. Dry samples aged 1 day, 1 week, 1 month, or 2 months were produced in this way. In the same way as for the glass substrate, a structural characterization was performed by AFM. The goal was to compare these samples with the samples prepared in situ, to follow their variation in time, and to provide information regarding their industrial applicability.

The same granular pattern was observed for the PE films prepared ex situ, demonstrating the lack of a dependence on the build-up procedure used (Figure 5). The grain diameter,



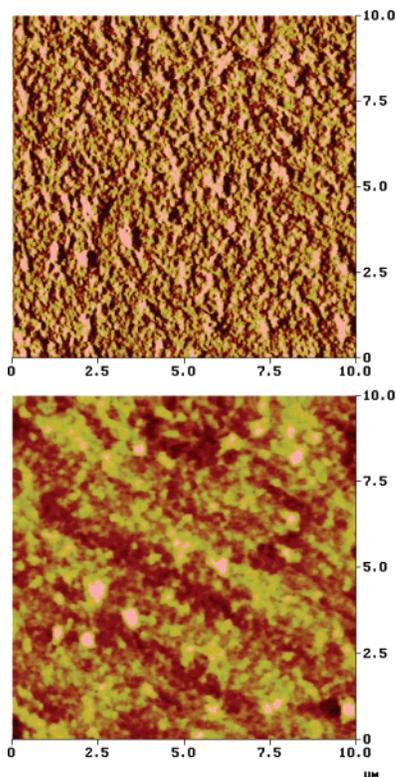
**Figure 4.** AFM deflection images of Ti substrate and PE layers on Ti (in situ measurements). (A) Bare Ti,  $z = 500$  nm; (B) (PLL/PGA)<sub>6</sub> multilayer,  $z = 800$  nm; and (C) (PLL/PGA)<sub>8</sub> film,  $z = 1.5$   $\mu$ m.

$303 \pm 89$  nm, was not significantly different from that obtained on glass.

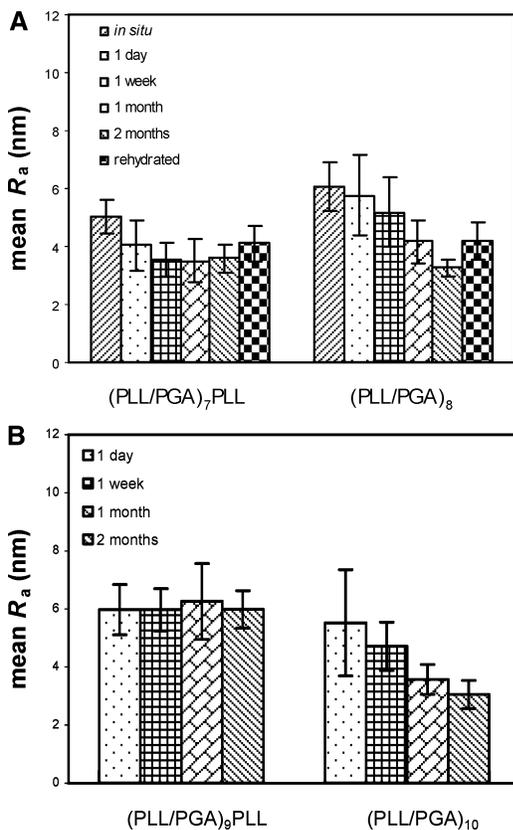
Figure 6A presents the mean roughness values for (PLL/PGA)<sub>7</sub>PLL and (PLL/PGA)<sub>8</sub> films, including the data on the in situ, ex situ, and rehydrated samples. For in situ deposition, the roughness for (PLL/PGA)<sub>7</sub>PLL was  $5.0 \pm 0.6$  nm, whereas for (PLL/PGA)<sub>8</sub> it was  $6.1 \pm 0.8$  nm.

PE films on a Ti substrate displayed a better stability in time up to 2 months as compared with glass substrate (Figures 6A and 3). However, the 1-month-old samples of (PLL/PGA)<sub>7</sub>PLL and (PLL/PGA)<sub>8</sub> revealed almost the same roughness on the two different substrates.  $R_a$  for the (PLL/PGA)<sub>7</sub>PLL film on glass was  $3.3 \pm 1.3$  nm, whereas that on the Ti substrate was  $3.5 \pm 0.8$  nm.

Figure 6B presents separately the mean roughness of thicker PE films (dry (PLL/PGA)<sub>9</sub>PLL and (PLL/PGA)<sub>10</sub>) deposited ex situ on a Ti substrate. For the PLL-ended films, a significant (almost double) increase in  $R_a$  was found for all of the dry samples: the typical  $R_a$  for a 1-week-old (PLL/PGA)<sub>7</sub>PLL sample was  $3.6 \pm 0.6$  nm, whereas that for the (PLL/PGA)<sub>9</sub>PLL film was  $5.9 \pm 0.7$  nm. In contrast for the PGA-ended films, there was no such accentuated variation in roughness. The present data do not yield any explanation for this difference; however, the small error bars prove that there is a real increase in  $R_a$  due to the PLL. Additional studies are needed to explain this phenomenon.

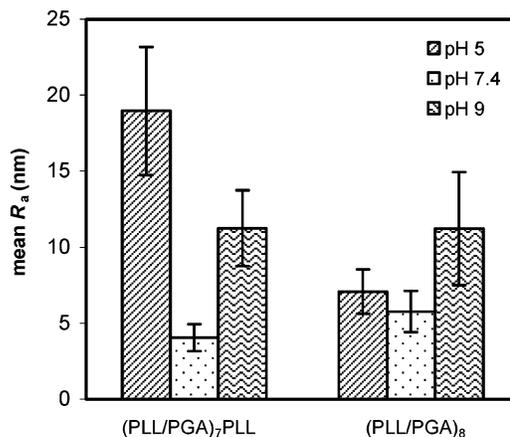


**Figure 5.** AFM deflection and height images of a (PLL/PGA)<sub>9</sub>PLL film on a Ti substrate, 1-day-old sample, immersion build-up technique.



**Figure 6.** Mean roughness values as a function of layer number and time for polypeptide films on a Ti. (A) (PLL/PGA)<sub>7</sub>PLL and (PLL/PGA)<sub>8</sub> films, rehydration after 6 weeks. (B) Dry samples of (PLL/PGA)<sub>9</sub>PLL and (PLL/PGA)<sub>10</sub> films.

Finally, when the 6-week-old samples were rehydrated, their roughnesses almost attained the in situ values: for (PLL/



**Figure 7.** pH dependence of mean roughness values for polypeptide films on a Ti substrate (1-day-old samples).

PGA)<sub>7</sub>PLL,  $4.1 \pm 0.6$  nm was measured, and for (PLL/PGA)<sub>8</sub>,  $4.2 \pm 0.7$  nm was measured.

**3.4. Structural Characteristics of PE Multilayers on a Ti Substrate as a Function of pH.** Since the degree of ionization of the PLL/PGA polypeptides is pH-sensitive, it may be expected that this parameter modulates the molecular organization of the films by altering the charge of both the polyanion and polycation. Weak PEs exhibit large variations in layer thickness and loops and tails above or below their  $pK_a$  as a result of incomplete charge compensation.<sup>22</sup> Morphological differences are observed when PLL/PGA films are deposited on a glass surface previously coated with strong PEs at pH 7.4 and pH 8.5.<sup>8</sup> We were interested in following the pH dependence of the PLL/PGA film structures by AFM, after direct adsorption on a Ti surface. Accordingly, films were deposited ex situ on Ti at pH 5, 7.4, and 9. Analysis of the AFM images revealed a significant effect of pH on the roughness of the (PLL/PGA)<sub>7</sub>PLL and (PLL/PGA)<sub>8</sub> films: the values generally increased at acidic or basic pH (Figure 7). There was also a noteworthy difference depending on the outermost layer of the PE film. When the outermost layer was PLL, high roughness values of  $18.9 \pm 4.2$  and  $11.2 \pm 2.5$  nm were obtained at low pH and high pH, respectively. In contrast, for the layers ending in PGA,  $R_a$  at pH 5 was much smaller, around  $7.1 \pm 1.5$  nm, whereas at pH 9 there was no difference as compared with the PLL-ending film.

The high roughness values observed at acidic or basic pH are related to the degree of ionization of PGA and PLL. The  $pK_a$  values of PGA and PLL in aqueous solution are 4.9 and 9.8, respectively.<sup>23,24</sup> At high pH, higher amounts of the only partly ionized PLL molecules are needed to neutralize the charges of the fully ionized PGA molecules. At low pH, where the PLL is totally ionized, and the PGA is only partly ionized, the same interactions govern the build-up of the films as at high pH, but in the opposite sense. These processes lead to the observed increase in roughness. Our results are in good accordance with the formation of thicker layers at acidic or basic pH, as observed earlier.<sup>8</sup>

**4. Conclusions**

The final thickness of the (PLL/PGA)<sub>8</sub> film in situ was found to be about 70 nm by OWLS. Structural characteriza-

tion of the (PLL/PGA)<sub>n</sub> self-assembled layers adsorbed on glass and Ti substrates deposited irreversibly in two ways: in situ or ex situ was achieved by means of AFM investigations.

The films on both glass and Ti substrates exhibited granular patterns with a grain diameter of  $270 \pm 87$  nm on glass and  $303 \pm 89$  nm on Ti, independently of the build-up procedure used.

With glass as the substrate, the roughness values of the dried samples decreased as compared with those obtained for the PE films built in situ, depending on the time that had passed. This effect was much less accentuated for the Ti surfaces, and the PE films revealed a better stability in time up to a 2 month period relative to those on glass. The roughness values for the dry PE films built ex situ on Ti were closer to the in situ values. However, on Ti a significant increase in  $R_a$  was observed for the (PLL/PGA)<sub>9</sub>PLL film. PLL seems to be the key factor in this growth: when PGA was added in the next layer,  $R_a$  resumed a value of about 3.5 nm.

Upon the rehydration of dry samples, the roughness values almost attained those measured in situ for both glass and Ti. For a Ti substrate, a significant pH effect was observed. The high roughness values observed at acidic or basic pH are related to the different degrees of ionization of PGA and PLL.

The technique of alternating adsorption of the polypeptides PLL and PGA proved to be a successful method with which to coat the Ti surface of a possible implant material and is accompanied by a number of advantages. It can be automated, it uses aqueous solutions of biodegradable polypeptides, and it can provide films with controlled surface charge and finely tuneable thickness and roughness. The demonstrated time stability and good rehydration capability of PLL/PGA multilayer films built on Ti by immersion are of prime importance as concerns their biomedical applications, providing stable coatings at the solid–liquid interface.

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